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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 39/05, C07K 7/02, 17/08 — A1

(11) International Publication Number: **WO 90/11778**

(43) International Publication Date: 18 October 1990 (18.10.90)

(21) International Application Number: PCT/US90/02039

(22) International Filing Date: 10 April 1990 (10.04.90)

(30) Priority data:
336,852 12 April 1989 (12.04.89) US

(60) Parent Application or Grant
(63) Related by Continuation
US 336,852 (CIP)
Filed on 12 April 1989 (12.04.89)

(71) Applicants *(for all designated States except US)*: THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021 (US). NEW YORK UNIVERSITY [US/US]; 70 Washington Square South, New York, NY 10012 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : TAM, James, P. [US/US]; 500 East 63rd Street, New York, NY 10021 (US). ZAVALA, Fidel, P. [US/US]; 110 Bleeker Street - Apt. 23F, New York, NY 10012 (US).

(74) Agents: BURKE, Henry, T. et al.; Wyatt, Gerber, Burke & Badie, 645 Madison Avenue, New York, NY 10022 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DENDRITIC POLYMER OF MULTIPLE ANTIGEN PEPTIDE SYSTEM USEFUL AS ANTI-MALARIAL VACCINE

(57) Abstract

Multiple antigen peptide systems are described in which a large number of each of T-cell and B-cell malarial antigens are bound to the functional groups of a dendritic core molecule providing a high concentration of antigen in a low molecular volume. The products elicit a very strong immunogenic response.

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"DENDRITIC POLYMER OF MULTIPLE ANTIGEN PEPTIDE SYSTEM USEFUL AS ANTI-MALARIAL VACCINE"

Vaccines often comprise an antigen on a natural carrier such as a protein, a carbohydrate, a lipid or a liposome. Such vaccines are useful and have been employed for many years. There are however a number of art recognized problems with them. Several of these problems are related to the carrier. Since the carriers are isolated from natural sources, they are often not of uniform quality. Additionally, despite expensive and arduous purification efforts, it is difficult, and often impossible, to provide products completely free of natural contaminants. Such contaminants may themselves be antigenic. They cause the undesirable side reactions often associated with the use of vaccines, particularly fevers and tissue swelling. Additionally, the concentration of antigen may vary from one batch to another because the amounts of antigen which react with the carrier or are absorbed on its surface are not uniform. This problem has markedly increased the difficulties of preparing suitable vaccines for protection against malaria.

Malaria is a particularly important target for synthetic vaccines, since it affects 200 million people worldwide and no immunoprophylaxis has yet been developed. It is known that protective immunity against rodent, simian and human malaria sporozoites can be induced by immunization with irradiated sporozoites. The major protein of the sporozoite is the circumsporozoite (CS) protein, and antibodies directed against the CS protein are known to neutralize the infectivity of parasites and inhibit their entry into the hepatocytes.

Thus, the CS protein has become an important target for the development of synthetic vaccines against the sporozoite stage of malaria. The immunodominant B-cell epitopes of the CS protein is contained within the repeating domain of the CS protein, a feature common to CS proteins of all malaria species. Mice immunized with a synthetic peptide, attached to tetanus toxoid as a protein carrier of this B-cell epitope, have been found to develop high antibody titers and resistance to challenge with 10^3 sporozoites. However, vaccination attempts in humans, using a similar approach, have failed to induce good antibody titers.

Recently, several T-helper cell epitopes of the CS protein of P. berghei (a rodent malaria) have also been identified (see Romero et al., Eur. J. Immunol. 18:1951, 1988). The identification of the B and T helper cell epitopes of the CS protein of P. berghei has now made it possible to incorporate these epitopes into one molecule in a specific and unambiguous manner using the MAP approach in which the epitopes are attached to a defined dendritic polymer, using the procedure developed by Tam and his coworkers as described in J. Biol. Chem. 263, 1719 (1988). In addition, T-cell epitopes of other malarial species have been identified: See, e.g., Sinigaglia, F. et al, Nature 336:778, 1988 (P. falciparum); Crisanti, A. et al., Science, 240:1324, 1988 (P. falciparum, blood stage); Kumar, S. et al., Nature 334:258, 1988 (P. falciparum sporozoites) etc; Good, M.S. et al, Science 235:1059-1062, 1987; Good, M.S., et al, Proc. Nat'l. Acad. Sci. 85:1199-1203, 1988; Sinigaglia, F., et al., Eur. J. Immunol. 18:633-636, 1988; and Guttinger, M., et al., EMBO J. 7:2555-2557, 1988.

Dendritic polymers are a new class of polymers. They are characterized by higher concentrations of functional groups per unit of molecular volume than ordinary polymers. Generally, they are based upon two or more identical branches originating from a core molecule having at least two functional groups. Such polymers have been described by Denkewalter et al. in U.S. Patent No. 4,289,872 and by Tomalia et al. in

several U.S. Patents including Nos. 4,599,400 and 4,507,466. Other polymers of the class have been described by Erickson in U.S. Patent 4,515,920. The polymers are often referred to as dendritic polymers because their structure may be symbolized as a tree with a core trunk and several branches. Unlike a tree, however, the branches in dendritic polymers are all substantially identical.

The products of this invention are based on such dendritic systems in which antigens are covalently bound to the branches which radiate from the core molecule. The system has been termed the multiple antigen peptide system and is sometimes referred to herein as MAPS. As will be apparent from the discussion hereinafter, some of the carrier or core molecules used to form the products of the invention are of a molecular weight such that they might not usually be regarded as polymers. However, since their basic structure is similar to dendritic polymers, it is convenient to describe them as such. Therefore, the term "dendritic polymer" will be sometimes used herein to define the polymeric substrates of the products of the invention. The term includes carrier molecules which are sufficiently large to be regarded as polymers as well as those which may contain as few as three monomers.

It has now been discovered that dendritic polymers can function usefully as carriers for a wide variety of antigens.

This invention will be better understood from a brief discussion of the structure of dendritic polymers.

Dendritic polymers are built upon a core molecule which is at lease difunctional. Each of the functional groups on the core molecule form at least two branches, the principal units of which are also at least difunctional. Each difunctional unit in a branch provides a base for added growth.

The system can be better visualized by reference to specific molecules. If, for example, lysine with two amino groups is joined in a peptide bond through its carboxyl group to the amino group of alanine or glycine which may in turn be bound to a resin, the resulting molecule will have two free amino groups. This dipeptide may be regarded as the first

generation. It may be joined to two additional lysine molecules by the formation of peptide bonds to produce a second generation molecule with four free amino groups. The process can be repeated to form third, fourth or even higher generations of products. With each generation the number of free amino groups increases geometrically and can be represented by 2^n, where n is the number of the generation.

Although none of these compounds are of particularly high molecular weight, it is convenient to refer to them as dendritic polymers.

Fig. 1 shows a three generation dendritic polymer core molecule based on lysine in which each of the eight available amino groups are joined to a peptide antigen through a glycine linker molecule.

The same types of reactions can be carried out with aspartic or glutamic acid, both of which have two carboxyl groups and one amino group to produce polyaspartic or polyglutamic acids with 2^n free carboxyl groups.

The necessary chemistry for performing these types of synthesis is known and available. With amino acids the chemistry for blocking functional groups which should not react and then removing the blocking groups when it is desired that the functional groups should react has been described in detail in numerous patents and articles in the technical literature.

The dendritic polymers can be produced on a resin as in the well-known Merrifield synthesis and then removed from the polymer.

Tomalia utilized ammonia or ethylenediamine as the core molecule. In this procedure, the core molecule is reacted with an acrylate ester by Michael addition and the ester groups removed by hydrolysis. The resulting first generation molecules contain three free carboxyl groups in the case of ammonia and four free carboxyl groups when ethylenediamine is employed. Tommalia extends the dendritic polymer with ethylenediamine followed by another acrylic ester monomer, and repeats the sequence until the desired molecular weight is attained. It will, however, be readily apparent to one skilled in the art,

that each branch of the dendritic polymer can be lengthened by any of a number of selected procedures. For example, each branch can be extended by multiple reactions with lysine molecules.

Erickson utilized the classic Merrifield technique in which a polypeptide of substantially any desired molecular weight is grown from a solid resin support. As the technique is utilized for the preparation of dendritic polymers, the linking molecule which joins the polymer to the resin support is trifunctional. One of the functional groups is involved in the linkage to the resin, the other two functional groups serve as the starting point for the growth of the polymer. The polymer is removed from the resin when the desired molecular weight has been obtained. One standard cleavage procedure is treatment with liquid hydrogen fluoride at 0°C for one hour. Another, and more satisfactory procedure, is to utilize a complex of hydrogen fluoride and dimethylsulfide (HF:DMF) as described by Tam et al. in J. Am. Soc. (1983) 105: 6442. This procedure greatly minimizes side reactions and loss of peptide.

Denkewalter, in one example of his process, utilizes lysine as the core molecule. The amino groups of the core molecule are blocked by conversion to urethane groups. The carboxyl group is blocked by reaction with benzhydrylamine. Hydrolysis of the urethane groups generates a benzhydrylamide of lysine with two free amino groups which serve as the starting points for the growth of the dendritic polymer.

This brief outline of three of the available procedures for producing dendritic polymers should be adequate to teach those skilled in the art the basis principles of the current technology. They will also teach the skilled artisan the salient features of the polymers, one of the most important of which is that the polymers provide a large number of available functional groups in a small molecular volume. The result is that a high concentration of antigens in a small volume can be achieved by joining the antigen to those available functional groups. Moreover, the resulting molecular product contains a high proportion of antigen on a relatively small carrier. This

is in contrast to conventional products used as a basis for vaccines. These conventional products often are composed of a small amount of antigen on a large amount of carrier.

Other important features of the dendritic polymer as an antigen carrier are that the exact structure is known; there are no contaminants which may be themselves antigenic, produce tissue irritation or other undesirable reactions; the exact concentration of the antigen is known; the antigen is symmetrically distributed on the carrier; and the carrier can be utilized as a base for more than one antigen so that multivalent vaccines can be produced. The principal advantage of the MAPS technique as the basis for malarial vaccines of this invention is that unlike previous systems using natural carriers such as keyhole limpet hemocyanin, tetanus toxoid and bovine serum albumin, the carriers of this invention are fully defined chemical entities on which the antigens are dispersed in known concentrations. Additionally the antigen comprises a large part of the molecule not a relatively small and undefined proportion of the molecule as in the case of natural carriers.

For the vaccines of this invention, it is preferred that the core molecule be a naturally occurring amino acid such as lysine so that it can be dealt with by the body following the usual metabolic pathways. However, as will be explained more fully hereinafter, amino acids which are not naturally occurring, even those which are not alpha-amino acids can be employed. The acids, or any other asymmetric molecules used in building the core molecule can be in either the D or L form.

Although the dendritic polymers have been principally described hereinabove as polyamide polymers, it will be readily apparent that the carriers of this invention are not limited to dendritic polyamides. Any of a wide variety of molecules having at least two available functional groups can serve as core molecules. Propylene glycol, for example, can serve as the basis for a polyester dendritic polymer. Succinic acid with selected glycols or amines can serve as a core molecule to generate polyesters or polyamides. Diisocyanates can be used to generate polyurethanes. The important point is that the

core molecule has at least two available functional groups from which identical branches can be generated by sequential scaffolding-type reactions with additional molecules also having at least two available functional or anchoring groups on each branch. In the most simple case in which the core molecule has two available functional groups and each succeeding generation has two available functional groups, the number of anchoring sites to which malarial-origin T-cell and B-cell antigens employed in this invention can be anchored is expressed by $(2)^n$ where n is the number of the generation.

For a more complete discussion of the chemistry of dendritic polymers attention is directed to Tamalia et al., Polymer Journal 17 (1), 117 (1985), Akaroni et al, Marcomolecules 15, 1093 (1982), and the following United States Patents:

4,289,872	4,558,120
4,376,861	4,568,737
4,507,466	4,587,329
4,515,920	4,599,400
4,517,122	4,600,535

All cited patents, patent applications and references are incorporated by reference in their entirety.

THE INVENTION

This invention in its presently preferred embodiments provides a multiple antigen peptide system comprising a dendritic polymer base with a plurality of anchoring sites covalently bound to antigenic T-cell and B-cell epitopes of malarial proteins such as the CS protein such that the resulting construct bears both T and B epitopic peptides. The polymers comprise a central core molecule having at least two functional groups to which molecular branches having terminal functional groups are covalently bound. The terminal functional groups on the branches are covalently bonded to the epitopic peptides. The antigenic molecules are principally described herein as peptide antigens, but they are not limited to peptide antigens or even to antigens. Thus, peptides that are not antigenic by themselves may be rendered antigenic when bound to

the core molecule.

The selected antigen may be separately synthesized (by synthetic methods, including but not limited to recombinant DNA techniques, as is now well-known in the art) or otherwise obtained and joined to the carrier. Preferably, the antigen may be synthesized on the carrier by extending each branch of the polymer utilizing known peptide synthesis techniques.

Fig. 1 shows the structure of a dendritic polymer which may be employed in the practice of this invention. As will be seen, it is a three generation dendritic polylysine product. It may be produced by a conventional solid phase techniques by generating the polymer on a Pam or a Pop resin. See Mitchell et al., J. Org. Chem. (1978) 43, 2845 and Tam et al., J. Am. Chem. Soc., (1960) 102 6117. The polymer is then cleaved from the resin using, preferably HF:DMS. The dendritic polylysine, as shown, was built from a glycine linker originally joined through a benzyl linker to the resin. Other linkers such as alanine can be employed. Of course, the linker can be omitted, or a plurality of linker molecules can be utilized.

Fig. 1 shows a dendritic polymer each molecule of which carries eight peptides some of which represent T-cell epitopic peptides and others B-cell epitopic peptides of a Plasmodium species responsible for malaria, e.g., Plasmodium berghei, Plasmodium falciparum or Plasmodium vivax, P. yoelii, P. malariae, P. ovale, P. cynomolgi, P. knowlesi; etc. joined directly to each of the available functional groups on each terminal lysine moiety. It is preferable that the B- and T-epitopes on the polymer are of the same malarial species. The the present invention is not limited to polymers bearing only one T- and B-epitope combination from a single species. For example, MAPS bearing simultaneously T- and B-epitopes from P.vivax CS protein and T- and B-epitopes from P.falciparum CS protein are within the scope of the invention. In addition, the ability of a peptide to function as a T-helper epitope is not necessarily dependent upon the copresence of a B-cell epitope from the same malarial species. Hence, cross-species combinations of T-helper and B-cell epitopic peptides are also

contemplated. When the selected epitopic structures are relatively short, e.g. 6 to 14 residues, it has been observed that it is best to extend the polylysine by a linker such as a simple tri- or tetrapeptide of glycine, alanine or beta-alanine. However, for antigenic peptides with more than 14 residues, the linker is normally unnecessary.

This invention has been described for convenience, principally as applied to products built on lysine as the core molecule. In fact lysine and lysine like molecules such as ornithine, nor-lysine and beta-amino alanine are preferred molecules for building the products of this invention because they are relatively easy to obtain, they are easy to work with and they afford good yields.

Such molecules can be represented by the general formula:

$$H_2N - (CH_2)_x - \underset{H}{C} - (CH_2)_z\ COOH$$
$$\qquad\qquad\quad (CH_2)_y\ NH_2$$

wherein x, y and z are integers from 0 to 10, preferably 0 to 4 provided that at least one of them is 1 and the amino groups cannot be attached to the same carbon atom. In the most preferred molecules the total of x, y and z is from 2 to 6 and the amino groups are separated by at least two methylene groups.

Other preferred core molecules include ethylene diamine and like molecules with longer chains such as propylene diamine and butylene diamine. Such molecules may be represented by the general formula:

$$H_2N\ CH_2\ (CH_2)_n - CH_2\ NH_2$$

wherein n is an integer from 0 to 10, preferably 0 to 3.

Of course, ammonia can also be employed as the core molecule.

The development of synthetic vaccines against a large number of diseases has recently been greatly accelerated because of the recognition that a vaccine need not be based on a native protein, but may be based on a low molecular weight segment of the native protein. These segments, normally called

immunogenic determinants or epitopes are capable of stimulating the production of antibodies which will protect against infection by sporozoites bearing the native protein antigen and in turn introduced in the mammalian host by the bite of a mosquito vector.

This invention is concerned with malarial-origin T- and B-cell epitopic peptides such as those described by Romero, et al., Loc. cit. which is incorporated herein by reference. By way of nonlimiting example, some of the P. berghei T-cell epitopic peptides are:

	Designation
YNRNTVNRLLAD	1
59 69	
NEKIERNNKLKQP	N
80 92	
NDDSYIPSAEKI	3
249 260	
KQIRDSITEEWS	B-4
265 276	
GSGIRVRKRKGSNK	5
283 296	
SSIFNIVSNSLG	6
317 328	
NEKIERNNKLKQPDPPPPNPNDPPPPNPND	N+17.1
KQIRDSITEEWSDPPPPNPNDPPPPNPND	B-4+17.1

The last two antigens N+17.1 and B-4+17.1 represent a combination of T-cell epitopes N or B-4 with a B-cell epitope 17.1. The epitope 17.1 and its preparation are described in Zavala et al., J. Exp. Med., 166:1591, 1987, which is incorporated by reference. It should be noted that in the case of circumsporozoite protein, the B-cell epitope (which happens to be the immunodominant epitope) is repetitive in nature, e.g., $(DPPPPNPN)_x$ for P. berghei; $(DRAAGQPAG)_x$ or $(DRADGQPAG)_x$ or combinations of the two for P. vivax; $(NANP)_x$ for P. falciparum, $(QAQGDGANAGQP)_x$ for P. knowlesi, etc. wherein x is at least 2 for at least some malarial species. Repeats of cyclic permutations of these minimum repeating units will also yield

B-cell epitopic peptides, e.g. $(PNAN)_x$.

Some of the antigenic peptides which are currently available either commercially or by known synthetic or isolation techniques are listed in Table 1, below. The table lists the peptides which are segments of proteins associated with the disease or pathogen identified in the second column. The references identify the publications which describe the peptides and how to obtain them. The conventional abbreviations are used for the amino acids.

TABLE 1

PEPTIDE SEQUENCES SUITABLE FOR DEVELOPMENT OF VACCINES USING MAPS

	Peptide	Pathogen/Disease (protein)	Ref
A.	H-(Asn-Ala-Asn-Pro)$_n$-CH n>3	Malaria, CS protein of P. falciparum	1
B.	H-(Gly-Asp-Arg-Ala-Asp-Gly Gln-Pro-Ala)$_n$-OH n>2	Malaria, CS protein of P. vivax	2
C.	Glu-Gln-Asn-Val-Glu-His-Asp-Ala	Malaria, Pf 155 of P. falciparum	3
D.	Asn-Ala-Glu-Asn-Lys-Glu-Glu-Leu-Thr-Ser-Ser-Asp-Pro-Glu-Gly-Gln-Ile-Mat	Malaria, Marozoite surface protein of P. falciparum	4
E.	Asn-Ala-Asn-Pro-Asn-Val-Asp-Pro-Asn-Ala-Asn-Pro	Malaria, CS protein of P. falciparum	5

1. Zavala, et al, Science 228:1436, 1985
2. McCutchan, et al, Science 230:1381, 1985; Arnot, D.E., et al, Science, 230:815 (1985)
3. Udomsangpetch, et al, Science 231:57, 1986
4. Ravetch, et al, Science 227:1593, 1984
5. Nardin, E.H. et al, Science 246:1603, 1989

In addition, malarial T-helper cell epitopic peptides can be identified, as described above in the references of Sinigaglia et al etc. Briefly, once the amino acid sequence of a malarial protein is known, peptides corresponding to fragments of the protein can be synthesized and injected in mammals. T-cells

can then be harvested from blood samples of the immunized mammals and incubated in vitro in the presence of the peptide used for immunization. Such peptide are considered T-helper cell epitopic peptides if the T-cells proliferate during such incubation in the presence of such a peptide. To demonstrate whether these T-cell peptides are T-helper peptides, they are tested for elicitation of antibodies to a B-cell epitope by covalently linking the T-cell and the B-cell epitopic peptide and using the thus formed conjugate for immunization.

In the foregoing description the letters have the same meaning as is employed by those skilled in the peptide arts. These are:

A-alanine	M-methionine
C-cystine	N-asparagine
D-aspartic acid	P-proline
E-glutamic acid	Q-glutamine
F-phenylalanine	R-arginine
G-glycine	S-serine
H-histidine	T-threonine
I-isoleucine	V-valine
K-lysine	W-tryptophan
L-leucine	Y-tyrosine

A particular advantage of this invention is that the dendritic polymer can serve as a carrier for two or more different malarial antigens. This is particularly useful for producing multivalent vaccines (i.e. vaccines directed against more than one malarial species) and/or for producing vaccines against different stages of the malaria parasite. Vaccines produced from antigenic products of the invention in which both T-cell antigens and B-cell antigens associated with malaria are joined to the dendritic polymer in any of the various configurations illustrated in a non-limiting fashion in Figure 2 are especially useful because they are capable of generating extremely high antibody titers.

It has been discovered that when the T- and B-cell epitopes of this invention are covalently bound to MAP substrates, the resulting products will elicit levels of antibody

response which are 10 to 100 fold greater than those obtained in the past with recombinant CS protein or irradiated sporozoites. It has been further observed that, in mice, the B-T monomeric di-epitope not supported on a MAP substrate, or a mixture of B-epitope MAP and T-epitope MAP produced very low antibody response and no protection. The presently most preferred embodiment of the present invention is one where both a T and a B epitopic peptide are linked in tandem on the same functional group of the dendritic polymer substrate.

The specifically selected B- and T-epitopes of this invention can be placed on the MAP substrate in a variety of different arrangements as shown in Fig. 2. The figure shows alternate arrangement for the B-epitope (open blocks) and the T-epitope (solid blocks) which for P. berghei include PPPPNPDPPPPNPND and KQIRDSITEEWS, respectively.

In Figure 2, T-(4) and B-(4) are monomeric maps with four branches but only one epitope (again the immunodominant B-epitope for the CS protein comprises at least two occurrences of the repetitive unit). T-(8) and B-(8) are similar, but with 8 branches. In T(8)B and B(8)-T, there are 8 T or B epitopes on the branches of the dendritic polymer and one B-epitope or T-epitope on the root of the polymer. BT-(4), TB-(4), BT-(8) and TB-(8) illustrate presently preferred products of the invention in which the epitopes are arranged in tandem.

Naturally, it will be apparent to those skilled in the art that many combinations and numbers of malarial T- and B-epitopes are contemplated herein and are fully within the scope of the present invention.

It is also possible to produce products of the invention in which the B- and T-epitopes are arranged alternatively on the branches, i.e., one branch has only B-epitopes, the other only T-epitopes. For instance, in Fig. 2, T/B(8) represents an eight branch dendritic polymer base with alternating T and B malaria antigens, within the scope of the invention; T/B(4) is similar except that the polymer base has only four branches.

This is accomplished utilizing the orthogonal protection method by employing a dendritic polymer based on a diamino

compound such as lysine in which the amino groups are blocked with different amino blocking groups, one of which is stable to acid hydrolysis, the other of which is stable to alkaline hydrolysis. (See, for example, the schematic representation of Fig. 2, E and F).

Fluorenylmethyloxycarbonyl (Fmoc) is a base labile protecting group and is completely stable to acidic deprotection. The t-butoxycarbonyl blocking group (Boc) is stable under mildly acidic conditions such as 50% trifluoroacetic acid. By choosing Boc-lys (Boc)-OH, Boc-lys (Fmoc)-OH, Fmoc-lys Boc)-OH or Fmoc-lys (Fmoc)-OH, it is possible to place one set of antigens on the alpha amino group of lysine and another on the omega amino group. Those skilled in the art of peptide synthesis can readily devise methods of achieving the same types of products using diverse blocking groups and other dendritic polymers.

It will be apparent to those skilled in the art that many variations of the structures shown and discussed herein are possible. For example, the dendritic polymer may have a structure in which segments are joined through a disulfide bridge. Such structures can be readily formed from dendritic polymers in which the root contains a protected cystine which is oxidized by a mild oxidizing agent such as molecular iodine.

As another example, referring to Fig. 1, the glycine at the root of the dendritic polymer, i.e., the free glycine could be joined to, or replaced with, a T- or B-malarial peptide antigen which may be the same or different from the other peptide antigens on the branches of the dendritic polymer molecule. The T- and B-peptide antigens themselves may serve as the residue to which other lysine or similar molecules may be attached to provide additional branches to which still additional peptide antigens, antibiotics or non-peptide antigens may be attached.

The products of this invention can be employed to produce vaccines useful to protect against malarial infections of mammals including humans using any of the procedures known to those skilled in the art. The products can, for example, be suspended in a pharmaceutically acceptable medium or diluent, such as inert oil, suitably a vegetable oil such as sesame,

peanut or olive oil. Alternatively, they can be suspended in an aqueous isotonic buffer solution at a pH of about 5.6 to 7.4. Typically, such solutions will be made isotonic with sodium chloride and buffered with sodium citrate-citric acid or with phosphate. The solutions may be thickened with a thickening agent such as methyl cellulose.

Vaccines may also be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder or an alkaryl polyether alcohol, sulfonate or sulfate such as a Triton.

Stabilizers such as sorbitol or hydrolyzed gelatin may also be added to any of the above described compositions. It is not unusual to incorporate an antibiotic such as neomycin or other anti-infective agents to prevent infection.

Because the products of this invention provide such high antibody titers, in many instances they will be employed without carriers or adjuvants. However, if an adjuvant is employed it may be selected from any of those normally employed to stimulate the immunogenic system of mammals. These include, for example, Freund's adjuvant (complete or incomplete), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), and mineral gels such as aluminum phosphate or alum; killed Bordetella, tetanus toxoid, diphtheria toxoid, muramyl dipeptide, aluminum hydroxide, saponin, etc., but as stated above, such adjuvants or carriers are not necessary when the polymeric substrate of the present invention is used. Freund's adjuvant is no longer used in vaccine formulations for humans or for food animals because it contains nonmetabolizable mineral oil and is a potential carcinogen. It can be used in vaccines for non-food animals. Mineral gels are widely used in commercial veterinary vaccines.

The vaccines of the invention may be defined as comprising a pharmaceutically acceptable carrier, of the general nature described above, together with an amount of an antigenic product of the invention , i.e., a selected T- or B-cell epitope which is sufficient to produce an immunological response, i.e., a

protective antibody response in a mammal. An effective amount may be very small. It will, as is known, vary with the antigen. The quantity which constitutes an effective amount may vary depending on whether the vaccine is intended as a first treatment or as a booster treatment.

The amount of MAP will vary depending upon the specific immunogen, the response it elicits in various subjects, and the presence or absence of heterologous carrier or adjuvant. Generally, amounts within the range from about 1 to about 1,000 micrograms of MAP are contemplated. Optimal amounts can be ascertained by routine experimentation involving measurement of antibody titers and other parameters of mammalian immune response, as is well-known in the art. Repeat immunizations are preferred.

It may be convenient to provide the products of this invention as lyophilized or freeze dried powders ready to be reconstituted with a pharmaceutically acceptable carrier just prior to use.

Additional information on vaccine preparations and protocols is well-known. See, for example, European Application No. A_1 191,748 of SmithKline Beckman published on August 20, 1986; European Patent Application No. A_1 192,626 of SmithKline Beckman et al. published August 27, 1986; U.S. Patent Nos. 4,693,994; 4,707,357; 4,735,799; and 4,767,622.

All cited patents, patent applications and literature are incorporated by reference in their entirety.

Thus, this invention also provides a method of providing immunity in a mammal against infection by a malarial organism which comprises administering to the mammal an immunogenically effective amount of a compound or composition comprising a malarial T- and B-peptide-bearing MAP, such an amount being effective to inhibit parasitemia in a mammalian host pursuant to infection by a malarial organism, preferably prior to exposure of the mammal to the malarial organism.

Also contemplated are vaccines useful for inhibiting malarial infection by the sporozoite or other stages of malaria, comprising an effective amount of an immunogenic compound

comprising a malarial-origin T- and B-peptide-bearing MAP, and, optionally, a pharmaceutically acceptable carrier or diluent.

It will be apparent to those skilled in the art that the products of this invention, once the concept is understood can be prepared by procedures well known to the skilled artisan. The Tam procedures described in Proc. Natl. Acad. Sci. USA, 85:5409, 1988, Prosnett et al, J. Biol. Chem., 263:1719, 1988; and Chenag et al, Proc. Natl. Acad. Sci. USA 86:4929, 1988, all of which are incorporated by reference are illustrative.

A few general observations applicable to the synthesis of MAPS will be of assistance to those skilled in the art. These are:

1. The syntheses generally require a long coupling time (2-4 hours).

2. Dimethyl formamide is generally a more suitable solvent than methylene dichloride.

3. The peptide resin should not be dried at any stage of the synthesis since resolvation is extremely difficult.

4. Coupling should be closely monitored for completion of the coupling by the quantitative ninhydrin method.

5. The MAPS is best cleaved from the resin by the improved acid deprotection method with either HF or TFMSA (Tam, et al., J. Am. Chem. Sic., 105:6442, 1983; and J. Am. Chem. Soc., 108:5242, 1986) in dimethyl sulfide to avoid strong acid catalyzed side reactions.

6. MAPS tend to strongly aggregate after cleavage from the resin support. Purification is best effected by extensive dialysis under basic and strongly denaturing conditions in a dialysis medium which is 8M in urea and mercaptoethanol to remove undesirable aromatic additives of the cleavage reactions such as p-cresol and thiocresol. Further purification, if desire, can be effected using high performance gel-permeation or ion exchange chromatography. In most cases the MAPS could be used directly without further purification.

Table I summarizes the results of several tests conducted to determine the efficacy of the products of this invention for eliciting an immunogenic response in mice. It will be

observed that the MAP based products of this invention have uniformly high antibody titers compared to irradiated sporozoite, recombinant CS protein or monomer BT peptide. It will be observed also that the response varies with the structure of the BT immunogens.

Table I. Comparison of antibody titers induced by different immunogens of P. berghei and assayed with the recombinant CS protein and sporozoites.

Immunogen	Antibody Response IFA titers Sporozoite	RIA titers rCS protein
sporozoite[a]	2,048	8,192
recombinant CS protein[b]	2,048	2,048
monomer BT peptide[c]	800	1,024
BT-MAP(4)[c]	128,000	408,000
TB-MAP(4)	32,000	400,000
BT-MAP(8)	24,000	100,000
TB-MAP(8)	64,000	400,000

a. Four mice of the H-2^a halotype (B10,A strain) were injected intravenously two doses of $1x10^5$ irradiated P. berghei sporozoites at two-week intervals. Sera were collected and pooled ten days after the last injection. Antibody titers expressed as the reciprocal of the highest positive serum solution were obtained by using glutaraldehyde-fixed P. berghei sporozoites in an indirect immunofluorescense assay (IFA) or the recombinant CS protein in a radioimmunoassay (RIA).

b. Four mice of the H-2^a halotype (A/J strain) were injected i.p. with 25 ug of the recombinant CS (rCVS) P. berghei protein, emulsified in CFA on day 0, and S.C. with 25 ug of the rCS protein in IFA on day 15. Sera were collected ten days later.

c. Five mice of the H-2^a halotype (A/J strain) were injected i.p. with each 50 micrograms of the peptide immunogens consisting of two occurrences of the repeating unit of P. berghei

CS protein immunodominant region and one occurrence of a P. berghei CS protein-derived T-cell epitope peptide. The immunization schedule and the assay methods were similar to those for the recombinant CS protein.

Upon challenge of the thus immunized mice with 2000 sporozoites each, the BT-MAP(4) produced complete protection (i.e., prevented parasitemia) in 80% of the mice; TB-MAP(4) protected 60% of the mice; BT-MAP(8) protected 50% of the mice; and TB-MAP(8) protected 60% of the mice.

MAPs according to the present invention may be synthesized as follows:

Some of the following abbreviations are used in the synthetic examples, below:

Boc - t-butoxycarbonyl
TFA - trifluoracetic acid
DMF - dimethylformamide
DCC - dicyclohexylcarbodiimide
Tos - tosyl
Bzl - benzyl
Dnp - dinitrophenyl
2ClZ - 2-chlorocarbobenzoxy
DIEA - diisopropoylethylamine
TFMSA - trifluormethylsulfonic acid
BSA - bovine serum albumin
HPLC - high performance liquid chromatography
TBR - tumor bearing rabbit
ATP - adenosine triphosphate
Dnp - dinitrophenyl.
ClZ - chlorobenzyloxycarbonyl
BrZ - bromobenzyloxycarbonyl
ELISA - enzyme linked immunoabsorbent assay

Example 1

General Methods for the Synthesis of Multiple-Antigen Peptides

The synthesis of an octabranched matrix core with peptide antigen was carried out manually be a stepwise solid-phase procedure [Merrifield, R.B. J. Am. Chem. Soc. (1963) 85,

2149] on Boc-beta-Ala-OCH_2-Pam resin with a typical scale of 0.5 g of resin (0.05 mmol and a resin substitution level of 0.1 mmol/g for the present synthesis but was somewhat lower when a higher branching of core lysinlyl matrix was used). After the removal of the Boc-group by 50% TFA and neutralization of the resulting salt by DIEA, the synthesis of the first level of the carrier-core was achieved using 4 molar excess of preformed symmetrical anhydride of Boc-Lys (Boc) (0.2 mmol) in DMF and was then recoupled via DCC alone in CH_2Cl_2. The second and third level were synthesized by the same protocol with 0.4 and 0.8 mmol respectively of preactivated Boc-Lys (Boc) to give the octabranching Boc-Lys(Boc)-core matrix. However, all subsequent couplings of the peptide-antigen sequence require 1.6 mmol of preactivated amino acids. The protecting groups for the synthesis of the peptide antigens were as follows: Boc group for the alpha-amino terminus and benzyl alcohol derivatives for most side chains of trifunctional amino acids i.e., Arg(Tos), Asp(OBzl), Glu(OBzl), His(Dnp), Lys(2ClZ), Ser(Bzl), Thr(Bzl), and Tyr(BrZ). Because of the geometric increase in weight gain and volume, a new volume ratio of 30 ml of solvent per g of resin was used. Deprotection by TFA (20 min) was preceded by two TFA prewashes for 2 min each. Neutralization by DIEA was in CH_2Cl_2 (5% DIEA) and there was an additional neutralization of DMF (2% DIEA). For all residues except Arg, Asn, Gln, and Gly, the first coupling was done with the preformed symmetric anhydride in CH_2Cl_2 and a second coupling was performed in DMF; each coupling was for 2 h. The coupling of Boc-Asn and Boc-Gly were mediated by the preformed 1-hydroxybenzotriazole ester in DMF. Boc-Gly and Boc-Arg were coupled with DCC alone to avoid the risk of formation of dipeptide and lactam formation, respectively. All couplings were monitored by a quantitative ninhydrin test [Sarin, V.K., et al Anal. Biochem. (1981) 117, 147] after each cycle, and if needed, a third coupling of symmetrical anhydride in DMF at 50° for 2 h was used [Tam, J.P. (1985) In "Proc. Am. Pept. Sympo., 9th" (C.M. Deber, K.D. Kopple and V.J.]. The synthesis was terminated with acetylation in acetic anhydride/DMF (3 mmol) containing 0.3 mmol of N,N-dimethylpyridine.

After completion of the MAPS, protected peptide-resin (0.3g) was treated with 1 M thiophenol in DMF for 8 h (3 times and at 50°C if necessary to complete the reaction) to remove the N^{im}-dinitrophenyl protecting group of His (when present), with 50% TFA/CH_2Cl_2 (10 ml) for 5 min to remove the N -Boc group, and with the low/high-HF method [Tam, J.P., Heath, W.F. & Merrifield, R.B. J. Am. Chem. Soc. (1983) 105, 6442] or the low-high TFMSA method [Tam, J.P. Heath, W.F. & Merrifield, R.B. J. Am. Chem. Soc. (1986) 108, 5242] of cleavage to give the crude MAPS. The crude peptide was then washed with cold ether mercaptoethanol (99:1, v/v, 30 ml) to remove p-thiocresol and p-cresol and extracted into 100 ml of 8 M urea, 0.2M dithiothreitol in 0.1 M Tris buffer, pH 8.0. To remove all the remaining aromatic byproducts generated in the cleavage step, the peptide in the dialysis tubing (Spectra Por 6,M.W. cutoff 1,000) was equilibrated in a deaerated and N_2-purged solution containing 8 M urea, 0.1 M NH_4HCO_3-$(NH_4)_2CO_3$, pH 8.0 with 0.1 M mercaptoethanol at 0°C for 24 h. The dialysis was then continued in 8M, and then in 2M urea, all in 0.1 M NH_4HCO_3-$(NH_4)_2CO_3$ buffer, ph 8.0 for 12 h and then sequentially in H_2O and 1 M HOAc to remove all the urea. The lyophilized MAPS was then purified batchwise by high performance gel-permeation or ion-exchange chromatography. All of the purified material gave a satisfactory amino acid analysis.

Example 2

Synthesis and Purification of (Asn-Ala-Asn-Pro)$_8$-MAP (NP-16 MAP), a Peptide Derived from the Sporozoite Stage of Plasmodium falciparum.

The peptide, (Asn-Ala-Asn-Pro)$_8$-Lys$_4$-Lys$_2$-Lys-OH was synthesized by the general procedure described in Example 1.

The synthesis was initiated with Box-Lys(Boc)-OCH_2-Pam-resin (a copoly(styrene-1%-divinylbenzene resin) at a substitution of 0.11 mmol/g of resin. The substitution was found to be 0.88 mmol/g after the sequential addition of three levels of Boc-Lys(Boc) to give an octabranching structure of [Boc-Lys(Boc)$_4$] [Lys(Boc)$_2$-Lys(Boc)-OCH_2-Pam resin. The synthesis continued with 2.5 g of resin in a modified Beckmann 990 synthesizer (Beckman

Instructions, Palo Alto, California). Synthesis was performed using a computer program that optimized all of the coupling steps. For example, the coupling of Boc-Ala and Boc-Pro were mediated by the symmetric anhydride method in a solvent ratio of CH_2Cl_2:dimethylformamide (1:3, v/v) to minimize aggregation and incomplete coupling. The coupling of Boc-Asn was by the performed 1-hydroxybenzotriazole active ester in the same solvent. Each amino acid underwent a double coupling protocol to maximize the coupling yield and essentially bring the reaction to >99.6% completion.

The protected peptide-resin was deprotected in portions. The initial deprotection was carried out with 1.57 g of dried peptide-resin in a reaction vessel and underwent the following procedure to remove the Boc-protecting group and other extraneous materials: CH_2Cl_2 (3 x 1 min wash); CF_3CO_2H-CH_2Cl_2(1:1, 3 x 2 min) and CF_3CO_2H (3 x 2 min wash) and then a cleavage reaction containing the following deprotecting reagents: trifluoromethanesulfonic acid:trifluoroacetic acid:tetrahydrothiophene: m-cresol (4:20:12:4, in ml) at 4°C for 3.5 h. The peptide released by the acidolytic cleavage of the sulfide-assisted cleavage procedure was collected and precipitated by ethyl ether (230 ml) prechilled to -30°C. The precipitate was centrifuged to a pellet and the ethyl ether was removed in vacuo. The peptide was then dissolved in 0.01M HOAc and dialyzed in 12 liters of 0.01M HOAc. The peptide was then lyophilized to dryness to obtain 60 mg of (Asn-Ala-Asn-Pro)$_8$0MAP. Hydrolysis of the resulting resin after cleavage showed that about 90% of the peptide had been cleaved from the resin support. The low yield was due to incomplete precipitation of the peptide by the ether. The same peptide-resin (1.0g) was also cleaved by HF:anisole (9:1, v/v total 10ml) at 0°C for 1 h to give 220 mg of MAP after extensive extraction with 10 to 100% HOACc and a crude yield of 33%. The dialysis was carried out with 10% OHAc.

The peptide after dialysis was then analyzed first by amino acid analysis (after hydrolysis by 6N HCl). The molar ratio of the MAP found was Asn:Ala;Pro;Lys: 1.97 (2): 1.03 (1):1 (1):0.26(0.22) which was in agreement with those expected

theoretical values shown in parenthesis.

Example 3

General Methods for the Synthesis of Di-epitope Multiple Antigen Peptides Containing Malarial-Provenance T-cell and B-cell antigens.

(a) Method A. Linking Two epitopes in Tandem.

The synthesis of di-epitope MAPS was accomplished manually by a stepwise solid-phase procedure on Boc-Ala-OCH_2-Pam resin (0.1 mmol of Ala is present in 1 g of resin) similar to those mono-epitope MAPS described in the previous examples. After the removal of the Boc group by 50% TFA and neutralization of the resulting salt by DIEA, the synthesis of the first level of the carrier core to form Boc-Lys(Boc)-Ala-OCH_2-Pam resin was achieved using a 4 mole excess of Boc-Lys(Boc) via DCC alone in CH_2Cl_2. The second and third level were synthesized by the same protocol, to give the octabranching Boc-Lys(Boc) core matrix. From this point onward, the synthesis of peptide antigens or two epitopes proceeded as those of the previous examples using the tertbutoxycarbonyl/benzyl protecting group strategy since they were arranged in tandem and were treated as if they are one antigen. Spacers such as tetra-peptide Gly-Pro-Pro-Gly are sometimes inserted between two peptide antigens to allow flexibility. After completion of the synthesis, the MAP-resin was treated with TFA to remove the N -Boc groups, then acetylated with 10% acetic anhydride/10% DIEA in Ch_2Cl_2, and finally cleaved with the low-high HF method to remove the MAP from the resin support. The crude peptide was then washed with cold ether/mercaptoethanol (99:1 vol/vol) to remove p-thiocresol and p-cresol, and extracted into 8 M urea in 0.1 M Tris.HCl buffer (pH 8.0). To remove the remaining aromatic by-products generated in the cleavage step, MAPs were dialyzed (Spectra Por 6, molecular weight cut off 1,000) in 8 M urea and then in 0.1 M acetic acid twice for 5-6 hours to remove the urea. The MAPs were lyophilized from H_O three times to remove acetic acid.

(b) Method B. Linking Two or More Epitopes by Alternating Branching of the Amino groups of Lysines

Because there are two amino groups in lysine and because these two amino groups could be protected selectively, the core matrix could be synthesized in such a way to produce that the N $-NH_2$ group is protected with the acid-labile Boc group and the N $-NE_2$ group is protected with the base-labile Fmoc (fluorenylmethoxycarbonyl) group, or vice versa, i.e. N $-NH_2$ group is protected by the Fmoc group, and the N $-NH_2$ group is protected by the Boc group. To achieve the synthesis of this core matrix using this selectivity, a core matrix containing N-NH_2-Boc and N $-NH_2$-Fmoc is illustrated. The synthesis of the core matrix was similar to those described in the previous examples using the Boc-Lys(Boc) for the branching for the first and second level. At the third level, Fmoc-Lys(Boc) was used for the Lys branching of the core to give for each Lys(Boc) and Fmoc-Lys end groups. The synthesis of the first epitope (or two epitopes in tandem) used the Boc/benzyl chemistry as described in the previous examples, but during this synthesis, neutralization time was reduced to 1 min to minimize the premature cleavage of the Fmoc group. The synthesis of the second epitope used the Fmoc/tertbutyl chemistry (i.e. the N $-NH_2$ group is protected with Fmoc and the side chain is protected with tertbutyl alcohol derived protecting groups) and started after the completion of the first epitope using the Boc-amino acid chain was assembled. The Fmoc-amino acids were used with the side chain protecting groups for the trifunctional amino acids as follows: Glu(OBu^t), Asp(OBu^t), Lys(Boc) Thr(Bu^t), Ser(Bu^t), Tyr(Bu^t), Arg(Pmz), His(Trt), Trp(For), and Cys(Bu^t). Repetitive deprotection of N-Fmoc was by 20% piperidine in dimethylformamide and was preceded by one piperidine prewash and the coupling was mediated with DCC:HOBut in DMF. After completion of synthesis, the MAP resin was treated with low-high HF to remove the peptide chains from the resin. The workup and purification was essentially the same as those described in the previous examples. The procedure for assembling the peptide chain using the Fmoc.tertbutyl chemistry was as follows: (1) 20 mil DMF (3 x 1 min); (2) 20 ml piperidine/DMF (1:1 vol/vol()1 min); (3) 20 ml piperidine/DMF (1:1 vol/vol) (10 min); (4) 20 ml DMF (3 x 1 min); (5) 20 ml

CH_2Cl_2(3 x 1 min); (6) 20 ml DMF (2 x 1 min); (7) amino acid (4 equiv) in DMF 5 ml (5 min), HOBt(4 equiv) in DMF, DCC(4 equiv) in CH_2Cl_2 were added for 2 h; (8) 20 ml DMF (4 x 2 min); (9) 20 ml CH_2Cl_2(2 x 2 min).

(c) Method C. Linking Two or More Epitopes via Disulfide Linkage of Two Preformed Heterologous MAPS.

To link two or more epitopes together via disulfide linkage of two preformed MAPS, a dipeptide such as Cys(Acm)-Ala is added at the carboxy terminus of the preformed MAPS as described in Example 3a or 3b. This could be achieved conveniently before the start of the synthesis of the core matrix by adding Boc-Cys(Acm) to the Boc-Ala-OCH_2-Pam-resin. After the formation of the dipeptide Boc-Cys(Acm)-Ala-OCh_Pam-resin, the synthesis of the core matrix, the incorporation of one or more peptide antigen(s) using the procedures described above proceeded to give the preformed MAPS containing a Cys(Acm)-Ala dipeptide COOH-tail. The Cys(Acm) is stable to the HF deprotection method. The preformed MAPS containing the COOH Cys(Acm)-Ala dipeptide tail were purified. The dimerization of two heterologous preformed MAPs was achieved by oxidation with I_2 to the disulfide, and which also concomitantly remove the Acm-group from the cysteinyl residue. A detailed procedure was as follows. To 1 mmol of MAP, the heterologous preformed di-epitope MAPs containing Cys(Acm) was dissolved in a de-aerated and N_2-purified 50% acetic acid solution at room temperature, 50 ml of a solution of I_2 in MeOH(1 M solution) was added batchwise for 1 hour at 0°C. The reaction was quenched by adding 1 M aqueous sodium thiosulfate (or ascorbic acid) until the yellow color was removed. MeOH was removed by dialysis in 0.1 acetic acid and the desired MAPs were purified by gel permeation chromatography, ion-exchange chromatography or reverse-phase high pressure liquid chromatography.

WHAT IS CLAIMED IS:

1. An antigenic product comprising a dendritic polymer having functional groups to which a plurality of both T-cell and B-cell epitopic peptide molecules selected from the group consisting of malarial B-cell and T-cell epitopic peptides are attached.

2. The product of claim 1 wherein at least one T-cell and B-cell epitopic peptide are attached in tandem to the same functional group.

3. The product of claim 1 wherein said T-cell and B-cell epitopic peptides comprise T- and B-cell epitopic peptides derived from the circumsporozoite protein of at least one species of malaria selected from the group consisting of P. berghei, P. knowlesi, P. yoeli, P. malariae, P. ovale, P. falciparum, and P. vivax.

4. The product of claim 3 wherein said B-cell epitopic peptides comprise amino acid sequences selected from the group consisting of

(a) $(NANP)_x$

(b) $(DRAZGQPAG)_x$ wherein Z is independently selected from A or D;

(c) $(QAQGDGANAGQP)_x$

(d) $(DPPPPNPN)_x$

(e) $(YAAA(A)_nGGG(G)_mN)_x$ wherein Y is D or G independently; and n = 0 or 1; and m = 0 or 1 independently;

(f) combinations of the foregoing;

(g) peptides consisting of cyclic permutations of each of the repeating units (a) through (e);

wherein x is an integer of at least 1; and the T-cell epitope is one or more T-cell epitopes derived from the CS protein of the same malarial species as the B-cell epitope.

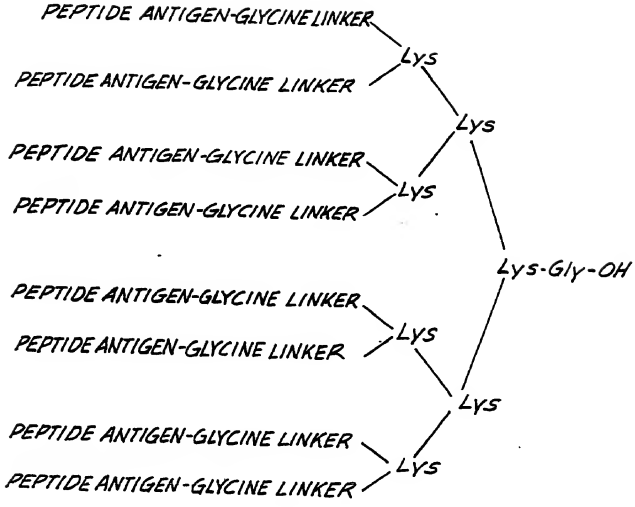
5. The product of claim 4 wherein a T-cell epitopic peptide is appended directly to a functional group of the dendritic polymer and the B-cell epitopic peptide derived from the same malarial species is appended to the other end of the T-

cell peptide, optionally via a linker.

6. The product of claim 4, wherein more than one T-cell epitopic peptide derived from the same malarial species is included along with at least one B-cell epitopic peptide derived from said species.

7. A vaccine against malaria comprising an immunogenically effective amount of the product of any one of claims 1-6.

8. A method for providing immunity against malaria in a mammal in need of such treatment comprising administering to said mammal an immunogenically effective amount of the product of anyone of claims 1-6.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Lys
Lys
Lys
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Lys
Lys-Gly-OH

FIG. 1

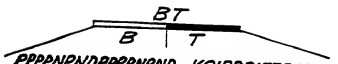


FIG.2 A

FIG.2B

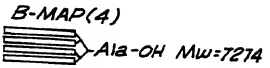


FIG.2C

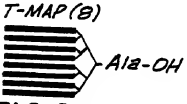


FIG.2D

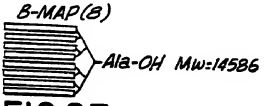
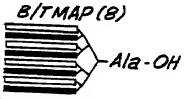


FIG.2E



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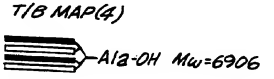

FIG.2G

FIG.2H

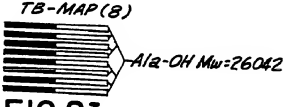

FIG.2I

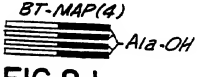

FIG.2J

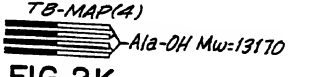

FIG.2K

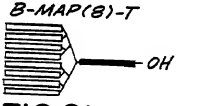

FIG.2L

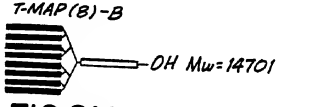

FIG.2M

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/02039

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 39/05; C07K 7/02, 17/08

U.S.Cl.: 424/88; 530/330, 324, 325, 326, 327, 328, 329, 403

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched [7] Classification Symbols
U.S.Cl.	424/88; 530/324, 325, 326, 327, 328, 329, 330, 332, 345, 403, 404, 405, 406, 806, 807, 822

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

computer search files: APS, CAS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,289,872 (DENKEWALTER et al) 15 September 1981, see column 3-4 and calim 5.	1-8
Y	US, A, 4,707,357 (DAME et al) 17 November 1987, see columns 3 (lines 40-44), 4-6 and claims 21-25.	4, 7, 8
Y	US, A, 4,713,366 (STEVENS) 15 December 1987, see columns 3-6, 34, 35, 39 and 48.	5, 7, 8
Y	Macromolecules, volume 15, issued July-August 1982, Aharoni et al., "Size and Solution Properties of Globular tert-Butyloxcarbonyl-poly (α,ε-L-lysine)," See pages 1093-1095	1-6

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search: 13 July 1990

Date of Mailing of this International Search Report: 28 AUG 1990

International Searching Authority: ISA/US

Signature of Authorized Officer: Kay K. Kim

NGUYEN, LONG-HO
INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (Rev.11-87)